EuroFlow Standard Operating Protocol (SOP) for

Bulk Lysis for MRD panels

Version 1.1
6 May 2014

For complementary information or questions, please go to www.euroflow.org

or contact Juan Flores-Montero at jflores@usal.es

Bulk Lysis protocol for MRD panels

Content:

Introduction	3
Ammonium Chloride lysing solution options	4
Sample processing protocol	5
Appendix	9

Introduction

- Valid for bone marrow (BM) or peripheral blood (PB) samples.
- Assess the cell concentration before any manipulation by an automated cell counter or manual counting.
- In order to reach the intended proposed sensitivity (i.e. 10^{-5}), staining of at least 10 x 10^6 nucleated cells for each MRD tube and the systematic acquisition of ≥ 5 x 10^6 cells are required conditions.

1. Ammonium Chloride lysing solution options:

The following options have been evaluated and found to perform equivalently

1.1. Commercial Ammonium Chloride (NH_4Cl) based lysing solution from BD (Pharm Lyse, cat reference: 555899). This lysing solution is available as a 10X stock solution and must be diluted prior to use to 1X in distilled water (dH_2O).

1.2. In-house prepared Ammonium Chloride lysing solution (See Appendix).

Please, take care that:

1.2.1. Follow the attached recipe for preparation

1.2.2. pH is stable at 7.4; and

1.2.3. A working solution is freshly prepared (<7 days).

2. Sample processing protocol:

2.1. Transfer the sample containing at least 10 x 10^6 nucleated cells for each MRD tube to be stained to a 50 mL Falcon tube (in case of BM, previous passes of the sample through a 25g-needle are not usually required). Do not use more than 2 mL of sample per 50 mL of lysing solution. If more sample need to be processed (i.e. starting cells concentration is low), use several 50 mL tubes.

2.2. Fill the tube up to reach 50 mL volume with Pharm Lyse solution (diluted to 1X in dH_2O and at room temperature - RT) or in-house made NH_4CL working solution.

2.3. Mix well and incubate for 15 min in a roller or sample-shaker device.

2.4. Centrifuge at 800 g for 10 min and remove the supernatant using a Pasteur pipette or a vacuum system without disturbing the cell pellet. Alternatively, the supernatant can be decanted and drops attached to the rim of the tube can be sucked away using a paper towel. Typically, 300 µL of cell suspension should remain in the tube.

2.5. Add 2 mL of PBS + 0.09% of NaN_3 + 0.5 % of BSA and resuspend the cell pellet vigorously.

2.6. Complete the volume of the tube containing the cell suspension up to 50 mL final volume with PBS + 0.09% of NaN_3 + 0.5 % of BSA.

2.7. Mix well.

2.8. Centrifuge at 800 g for 5 min and remove the supernatant using a Pasteur pipette or a vacuum system, without disturbing the cell pellet. Alternatively, the supernatant can be decanted and drops attached to the rim of the tube can be sucked away using a paper towel.

2.9. Resuspend the cell pellet in 2 mL of PBS + 0.09% of NaN_3 + 0.5 % of BSA. Mix well and transfer this volume to a 5 mL polystyrene round-bottom Falcon tube ("FACS tube").

2.10. Wash the 50 mL Falcon tube with 2 mL of PBS + 0.09% of NaN_3 + 0.5 % of BSA more to recover cells that might have been left in the original tube. Add this volume to the 5 mL Falcon tube containing the rest of the sample transferred in step 2.9.

2.11. Centrifuge at 540 g for 5 min and remove the supernatant by decanting or using a Pasteur pipette. If the remaining cell volume is lower than 300 µL, PBS + 0.09% of NaN_3 + 0.5 % of BSA will be added to reach a volume of at least 300 µL.

2.12. In case multiple 50 mL tubes were used (because it was needed to lyse large sample volumes) the cell suspensions from the same sample should be combined at this

moment, before adjusting cell concentration. Try to keep the final volume low, so that, in case that cell concentration needs to be adjusted as indicated in the next step, it can be easily done by diluting with the recommended buffer.

2.13. Adjust the final cells concentration to 1 x 10^5 cells / μL, by resuspending the pellet with PBS + 0.09% of NaN_3 + 0.5 % of BSA.

2.14. Calculate 100 μL (i.e. 10 million cells) of the sample solution per each tube to be stained/acquired.

2.15. Staining steps for surface membrane markers only:

2.15.1. Calculate the total volume of surface membrane backbone (common) antibodies based on the number of tubes to stain and the specific titer for each one.

2.15.2. Pipette these antibodies in one tube (backbone tube).

2.15.3. Calculate the total volume of sample to be stained, also based on the number of tubes in the panel (100μL / 10 x 10^6 per tube).

2.15.4. Pipette this sample volume into the backbone tube.

2.15.5. Mix well.

2.15.6. Mix the correspondent amounts of the sample and backbone mix into the different tubes of the panel.

2.15.7. Add the appropriate volume of other antibodies directed against cell surface markers (except for the backbone markers), if necessary, use PBS+0.5% BSA+0.09% NaN_3 to reach a final volume of 200 μL per tube.

2.15.8. Mix well.

2.15.9. Incubate for 30 min at RT protected from light.

2.15.10. Add 2 mL of 1X FACS Lysing Solution (10X FACS Lysing Solution diluted 1/10 vol/vol in dH_2O; 10X FACS Lysing Solution from BD Bioscience, cat. Reference 349202).

2.15.11. Mix well.

2.15.12. Incubate for 10 min at RT protected from light.

2.15.13. Centrifuge for 5 min at 540 g.

2.15.14. Discard the supernatant using a Pasteur pipette or vacuum system without disturbing the cell pellet, leaving approximately 100 μL residual volume in each tube.

2.15.15. Add 2 mL of PBS+0.5% BSA+0.09% NaN_3 to the cell pellet.

2.15.16. Mix well.

2.15.17. Centrifuge for 5 min at 540 g.

2.15.18. Discard the supernatant using a Pasteur pipette or vacuum system without disturbing the cell pellet, leaving approximately 100 µL residual volume in each tube.

2.15.19. Resuspend the cell pellet in 500 µL PBS+0.5% BSA (without NaN_3).

2.16. Staining steps for combined staining of intracellular and surface membrane markers:

2.16.1. Stain surface membrane markers following the protocol as described from step 2.15.1 to 2.15.9.

2.16.2. Add 2 mL of PBS+0.5% BSA+0.09% NaN_3 to the cell pellet.

2.16.3. Mix well.

2.16.4. Centrifuge for 5 min at 540 g.

2.16.5. Discard the supernatant using a Pasteur pipette or vacuum system without disturbing the cell pellet, leaving approximately 100 µL residual volume in each tube.

2.16.6. Resuspend the cell pellet by mixing gently.

2.16.7. Add 100 µL of Reagent A (fixative; Fix&Perm, An der Grub, Vienna, Austria) and mix thoroughly.

2.16.8. Incubate for 15 min at RT protected from light.

2.16.9. Add 2 mL of PBS+0.5% BSA+0.09% NaN_3 to the cell pellet.

2.16.10. Mix well.

2.16.11. Centrifuge for 5 min at 540 g.

2.16.12. Discard the supernatant using a Pasteur pipette or vacuum system without disturbing the cell pellet, leaving approximately 100 µL residual volume in each tube.

2.16.13. Resuspend the cell pellet by mixing thoroughly.

2.16.14. Add 100 µL of Reagent B (permeabilizing solution; Fix&Perm).

2.16.15. Mix well.

2.16.16. Add the intracellular antibodies in the appropriate volume.

2.16.17. Mix well.

2.16.18. Incubate for 15 min at RT protected from light.

2.16.19. Add 2 mL of PBS+0.5% BSA+0.09% NaN_3 to the cell pellet.

2.16.20. Mix well.

2.16.21. Centrifuge for 5 min at 540 g.

2.16.22. Discard the supernatant using a Pasteur pipette or vacuum system without disturbing the cell pellet, leaving approximately 100 µL residual volume in each tube.

2.16.23. Resuspend the cell pellet in 500 µL PBS+0.5% BSA (without NaN_3).

2.17. Acquire the cells immediately after staining or (if not immediately acquired) store at 4°C (for 1h maximum) until measured in the flow cytometer.

2.18. Acquire tube completely at “medium” flow rate.

Appendix

NH_4Cl lysis buffer reagents:

1. Ammonium chloride NH_4Cl 53.49 g/mol
2. Potassium hydrogen carbonate $KHCO_3$ 100.12 g/mol
3. EDTA solution 0.5 M pH 8.0 (Fluka, BioChemika or similar product)
4. NaOH solution

Preparation

Stock solution

1. Bring 83g of NH_4Cl and 10 g of $KHCO_3$ into a cylinder of 1 liter.
2. Add 900 mL Milli-Q water and mix until all powder is dissolved (at RT)
3. Add 2 mL of EDTA solution and mix.
4. Check the pH of the solution. When needed adjust with NaOH to pH 7.4.
5. Adjust the volume to 1 L precisely with Milli-Q water.
6. Store this stock solution in a 1-L bottle in the refrigerator.

For 1 L NH_4Cl working solution

- Dilute 100 mL NH_4Cl stock solution into a 1-L cylinder with Milli-Q water
- Mix well (RT)

Storage and shelf life

- Store the NH_4Cl stock solution in the refrigerator (2-7°C). Shelf life is 6 months
- Store the NH_4Cl working solution at RT (18-25°C). Shelf life is 1 week.